PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the **PATENT APPLICATION** of:	
Qi et al.	
Application No.: 10/031,520	Our File: CSI3-PT001
Confirmation No.: 6223	
Filed: January 17, 2002	
For: ALGAE PROTEIN POLYSACCHARIDE EXTRACTION AND USE THEREOF	
Group: 1651	
Examiner: Prats, Francisco Chandler	

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

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Commissioner for Patents
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I, Quing Qi, declare, under the penalty of perjury, that:

1. I am a named inventor of the subject matter described and claimed in the above-identified patent application.

2. I have A UNIVERSITY DEGREE and 11 years of experience as a BIOCHEMIST.

3. I am familiar with the above-identified patent application and with the applied reference, Shinpo et al. (JP 58-128322).

4. The above-identified application discloses and claims, in part, the steps of: (a) dissolving a dry powdered Spirulina in 5-20 times water by weight and breaking the Spirulina cell walls; (b) heating a solution obtained from step (a)

at 60°-100°C, and cooling the heated solution to separate a liquid phase from the solution; (c) adjusting pH of said liquid phase to 3.8-4.2, and filtering said liquid phase to obtain a filtrate; and (d) adjusting the filtrate to pH 7, and concentrating the filtrate.

5. Shinpo does not disclose the step of selective precipitation at pH 3.8-4.2 to remove specific impurities.

6. I have found that selective precipitation of Spirulina at different pH levels results in the recovery of different proteoglcyan extracts having different biochemical characteristics.

7. As shown in Exhibit "A," Spirulina precipitated at three different pH levels (*i.e.* pH 4, pH 5-6, and pH 8-9) produced main peaks and retention times for the resulting proteoglycan extracts which varied greatly using HPLC analysis. *See* Exhibit "A" at 1.1.

8. As shown in Exhibit "A," Spirulina precipitated at three different pH levels produced different results in therapeutic efficacy of the resulting proteoglycan extracts using a pharmacodynamic assay. *See* Exhibit "A" at 1.2. As shown in the attached exhibit, some of the therapeutic effects decreased significantly, and even disappeared in direct correlation to the pH level at which the proteoglycan extracts were precipitated.

9. This, in my opinion, is an unexpected result that was not apparent from the cited prior art.

10. Shinpo does not disclose breaking the cellular walls of Spirulina to remove the active components from Spirulina cells. Rather, Shinpo discloses hot water treatment which the Examiner claims "would have been expected to have broken at least a few cell walls."

11. As shown in Exhibit "B," experiments were performed to compare the extracted components from Spirulina cells using hot water treatment and ultrasonic treatment. In each of the examples using ultrasonic treatment, the concentration of proteoglycan in the extracted product was significantly greater than that of saccharide and protein. Whereas, in each of the examples using hot water treatment in accordance with the cited prior art, the concentration of proteoglycan in the extracted product was less than the amount of saccharide and significantly less than the amount of protein extracted.

12. In addition, it has been found that the 50% inhibition concentration (IC_{50}) for proteoglycan extracts resulting from ultrasonic treatment is significantly lower than that of proteoglycan extracts using hot water treatment.

13. Based on the foregoing, breaking the cellular walls of Spirulina using a cellular wall breaking method according to the present invention, such as by ultrasonic treatment, rapid stirring, osmotic pressure changing lysis, or enzymolysis, permits the removal of a product from Spirulina cells having greater quantities of proteoglycan as opposed to hot water treatment.

14. This, in my opinion, is an unexpected result that was not apparent from the cited prior art.

15. I have been warned that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. § 1001 and may jeopardize the validity of the application or any patent issuing thereon.

FURTHER DECLARANT SAYETH NOT.

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Date: NOV. 28, 2004

Quing Qi

Exhibit A: Analysis of the extracts obtained at different pH

1.1. HPLC assay

The inventors employed Varian 5060 High Performance Liquid Chromatographer, ODS C18 4×150 mm column, and ethanol and water as flowing phase to perform HPLC assay at 40℃, and the ethanol proportion increased from 0% to 10% within 10 min.

Column temperature: 40℃

Flowing phase:

A: water 100% ⟶ 90% (10' linear gradient)

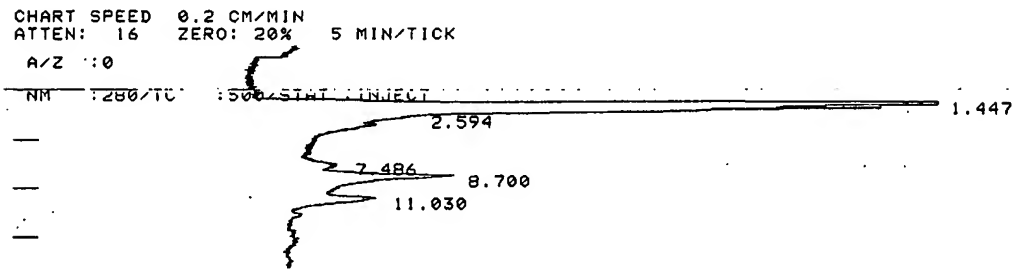
B: ethanol 0% ⟶ 10% (10' linear gradient)

Flow rate: 0.8 ml/min

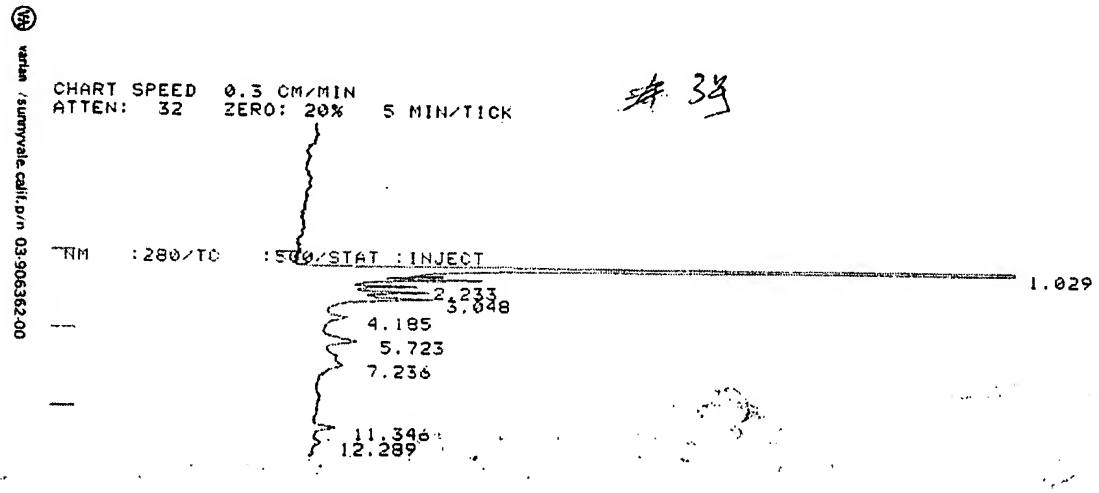
Detector: UV 280 nm

Sample volume: 50 μl (concentration: 0.8 mg/ml)

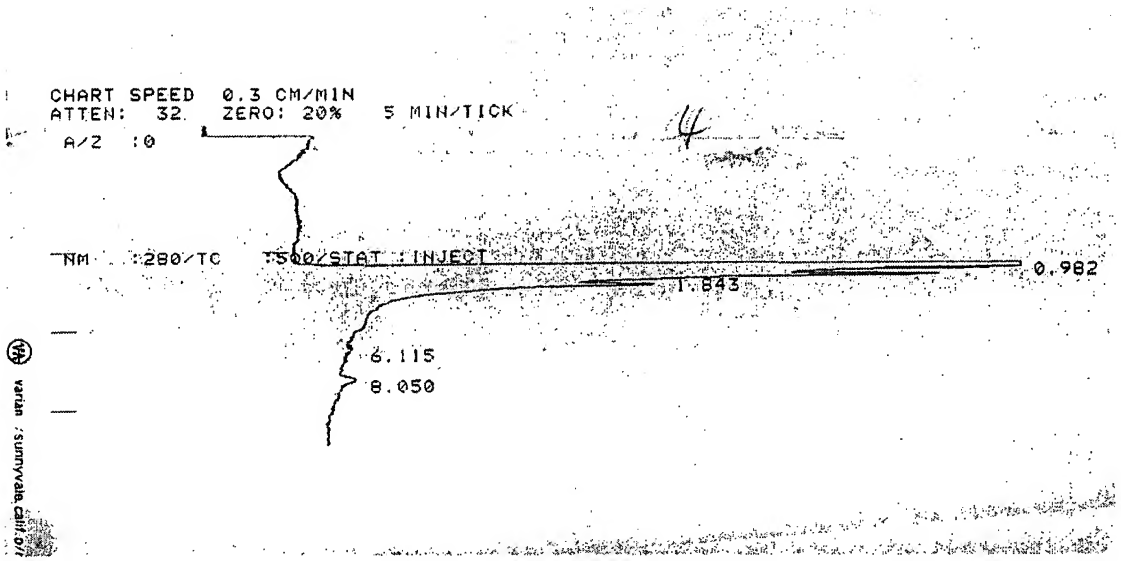
(1) The proteoglycan extract obtained at about pH 4



(2) The proteoglycan extract obtained at pH 5-6



(3) The proteoglycan extract obtained at pH 8-9



As can seen from the above profiles, under alkaline conditions, many peaks disappear, because some proteins have been denatured and precipitated, and ultimately absent in the sample.

1.2. Pharmacodynamic experiments for different proteoglycan extract obtained at different pH

Murine leukemia P-388 (a suspended tumor cell line) and Microculture Tetrozolium (MTT) were employed to perform pharmacodynamic experiments. Tumor cells at logarithm phase of growth were seeded in 90 μl/well into 96-well microplate depending on different tumor growth rate, and then the sample solution was added in 10 μl/well to the plate. From the results of preliminary experiments, samples were provided with 5 different concentrations for each cell line, and 3 double wells for each concentration. Further, the wells with no cell apoptosis were provided as a control. After the tumor cells were cultured at 37℃ in 5% CO_2 for 48 hr, MTT (Sigma) solution was added in 20 μl/well, continued to culture for 4 hr, and triplet solution (10% SDS-5% isobutanol-0.01mol/L HCl) was added in 50 μl/well, then cultured in CO_2 incubator overnight. The mixtures were measured at OD570 nm with ELISA reader. The inhibition rate of the test extract to tumor cell growth was calculated according to the following formula:

$$\text{Tumor inhibition rate} = \frac{\text{OD Value for control group} - \text{OD Value for treatment group}}{\text{OD Value for control group}} \times 100\%$$

50% inhibition concentration (IC50) was calculated with Logit algorithm. The experiment data are provided below:

(1) The proteoglycan extract obtained at about pH 4

Conc. (mg/ml) / Batch No.	1	0.5	0.25	0.13	0.063	IC50 (mg/ml)
I	83.8	72.7	54.1	40.0	20.7	0.21

II	82.1	72.8	60.2	30.5	16.9	0.23
III	87.8	73.3	68.7	45.9	32.5	0.14

(2) The proteoglycan extract obtained at pH 5-6

Conc. (mg/ml) / Batch No.	1	0.5	0.25	0.13	0.063	IC50 (mg/ml)
I	89.8	71.3	30.5	7.0	1.2	0.996
II	89.5	71.0	29.9	6.9	1.1	1.01
III	89.8	71.2	29.4	7.0	0.93	1.02

(3) The proteoglycan extract obtained at pH 8-9

Conc. (mg/ml) / Batch No.	1	0.5	0.25	0.13	0.063	IC50 (mg/ml)
I	45.3	13.7	0.8	0	0	No activity
II	30.8	6.8	0	0	0	No activity
III	57.1	19.4	2.1	0	0	No activity

Exhibit B:

1.1. Comparision of Ultrasonic treatment with hot water treatment

Components	Hot water treatment			Ultrasonic treatment		
	1	2	3	1	2	3
Saccharide	0.19	0.20	0.14	0.28	0.21	0.24
Proteoglycan	0.10	0.16	0.08	0.53	0.49	0.54
Protein	0.71	0.64	0.78	0.19	0.30	0.22

1.2. The pharmacodynamic assay data also demonstrated significant differences, i.e. IC50 for hot water treatment was greater than 4 mg/ml, and IC50 for ultrasonic treatment was less than 2 mg/ml.